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# A Differential Medium for the Enumeration of the Spoilage Yeast *Zygosaccharomyces bailii* in Wine

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## ABSTRACT

A collection of yeasts, isolated mostly from spoiled wines, was used in order to develop a differential medium for *Zygosaccharomyces bailii*. The 118 selected strains of 21 species differed in their origin and resistance to preservatives and belonged to the genera *Pichia, Torulaspora, Dekkera, Debaryomyces, Saccharomycodes, Issatchenkia, Kluyveromyces, Kloeckera, Lodderomyces, Schizosaccharomyces, Rhodotorula, Saccharomyces,* and *Zygosaccharomyces*. The design of the culture medium was based on the different ability of the various yeast species to grow in a mineral medium with glucose and formic acid (mixed-substrate medium) as the only carbon and energy sources and supplemented with an acid-base indicator. By manipulating the concentration of the acid and the sugar it was possible to select conditions where only *Z. bailii* strains gave rise to alkalinization, associated with a color change of the medium (positive response). The final composition of the mixed medium was adjusted as a compromise between the percentage of recovery and selectivity for *Z. bailii*. This was accomplished by the use of pure or mixed cultures of the yeast strains and applying the membrane filtration methodology. The microbiological analysis of two samples of contaminated Vinho Verde showed that the new medium can be considered as a differential medium to distinguish *Z. bailii* from other contaminating yeasts, having potential application in the microbiological control of wines and probably other beverages and foods.

The design of differential or selective media for preservative-resistant yeasts is an ongoing concern in the quality control of foods and beverages. This is particularly relevant for the case of *Zygosaccharomyces bailii,* which is a commonly encountered spoilage yeast. In the food and beverage industries this species is responsible for considerable economic losses *(3, 8)*. The low permeability of *Z. bailii* to weak acid preservatives at low pH values and its ability to metabolize acid compounds, even in the presence of glucose, are some of the physiological traits associated with its high tolerance to acidic environments *(2, 6, 7)*.

The aim of this work was to develop a differential medium for *Z. bailii*. The strategy followed was based on the use of a medium containing a mixture of glucose and formic acid, as sole carbon and energy sources with the incorporation of an acid-base indicator.

## MATERIALS AND METHODS

**Yeast strains and culture media.** The yeast strains selected for the present study (Table 1) were mostly isolated from spoiled wines and differed in their origin and resistance to preservatives. Cultures were maintained at room temperature on yeast extract peptone dextrose (YEPD) agar slants containing yeast extract (0.5%, wt/vol), peptone (1%, wt/vol), glucose (2%, wt/vol), and agar (2%, wt/vol), pH 6.9. The new *Z. bailii* differential (ZBD) medium *(5)* was based on a mineral medium supplemented with vitamins and oligoelements *(9)*, bromocresol green as acid-base indicator (0.005%, wt/vol), and a mixture of formic acid and glucose (mixed-substrate medium). The basic mineral medium containing the acid-base indicator was adjusted to pH 4.5, sterilized (121°C for 15 min), and cooled to 50 to 52°C. Subsequently, the filter-sterilized oligoelements, vitamins, and substrates, previously adjusted to pH 4.5 (NaOH 10 M), were added. A solid medium was prepared by the addition of agar (2.0%, wt/vol) to the basic mineral medium before sterilization.

*Z. bailii* selective agar (ZBA, pH 4.0) was prepared according to Erickson *(1)*, following correction of the published formula, as mentioned by Makdesi and Beuchat *(4)*. The Wallerstein laboratory nutrient (WLN, pH 5.5) and differential (WLD, pH 5.5) agars, both commercialized by Difco, were used for the field trial assays with contaminated wine samples.

**Development of the mixed-substrate culture medium.** After 2 days of growth on YEPD slants, a cell suspension in sterile deionized water was prepared in order to obtain an optical density ($OD_{640}$) between 0.7 and 1.0. Microplate wells containing 225 μl of mixed-substrate liquid culture medium were inoculated with 25 μl of the cell suspension. The inoculation of the solidified medium was performed by dropping 10 μl of the cell suspension onto the surface of an agar plate. The microplates were mechanically shaken at 180 rpm. Color change of the drops or suspensions was evaluated after incubation at 30°C for 48 h.

**Validation of the culture medium—recovery of *Z. bailii* cells.** The ZBD medium, containing 0.1% (wt/vol) glucose and 0.2, 0.3, 0.4, or 0.5% (vol/vol) formic acid, was compared with the formerly described *Z. bailii* selective medium (ZBA) *(1)* for the ability to recover *Z. bailii* cells. YEPD was used as a nonselective control medium for evaluating the percentage recovery. In order to simulate the yeast flora of a spoiled wine, several typical wine-contaminating yeast species, such as *Z. bailii, Saccharomyces cerevisiae, Pichia membranaefaciens,* and *Dekkera anomala,* were selected. These species were tested in pure or mixed cultures.

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TABLE 1. *Yeast strains used for the development of the differential culture medium*

| | |
|---|---|
| *Zygosaccharomyces bailii* | IGC:[a] 4267, 4806, 4227, 4531, T5167 |
| | ISA: 1023, 1024, 1025, 1031, 1095, 1148, 1206, 1212, 1214, 1265 |
| *Zygosaccharomyces bisporus* | IGC: T5335, 5336, 5337, 5381, 5382, 5383, 5384, 5385 |
| *Zygosaccharomyces rouxii* | IGC: 4194, 3691, 3693, 3694, 3701 |
| | ISA: 1220, 1552, 1553 |
| *Zygosaccharomyces florentinus* | IGC: 4169 |
| *Debaryomyces hansenii* | IGC: 2968 |
| | INETI: CL18 |
| *Dekkera anomala* | IGC: 5133, 5160, 5161 |
| *Dekkera bruxellensis* | IGC: 4179, 4801, 4808, 5162 |
| *Issatchenkia orientalis* | IGC: 2631, 3341, 3806, 5041, 5044, 5046 |
| *Kloeckera apiculata* | ISA: 1189 |
| *Kluyveromyces marxianus* | IGC: 2671, 2902, 3014, 3286, 3886 |
| *Lodderomyces elongisporus* | ISA: 1308, 1421 |
| *Pichia anomala* | IGC: 2495, 2505, 3294, 4121, 4380, 4554, 5008 |
| *Pichia membranaefaciens* | IGC: 2487, 2582, 3315, 3796, 4275, 4475, 4829, 4875, 5013, 5015, 5017, 5019, 5122 |
| *Rhodotorula mucilaginosa* | IGC: 4791, 5166 |
| *Saccharomyces bayanus* | IGC: 4565, T4456 |
| *Saccharomyces cerevisiae* | CBS: 1871-1, 1871-2, 5494, 5495 |
| | IGC: T2608, 3507-III, 3507, 2917, 3970, 3977, 4003, 4017, 4022, 4023, 4024, 4072, 4237, 4240, 4241, T4455, 4891 |
| *Saccharomyces pastorianus* | IGC: 4579, T4601 |
| *Saccharomycodes ludwigii* | ISA: 1083, 1088, 1089 |
| *Schizosaccharomyces pombe* | ISA: 1190, 1191, 1992, 1193 |
| *Torulaspora delbrueckii* | IGC: 4182, 2916, 3209, T2477 |
| | ISA: 1229, 1549 |

[a] ISA, Instituto Superior de Agronomia (Lisboa, Portugal); IGC, Instituto Gulbenkian de Ciências (Oeiras, Portugal); INETI, Instituto Nacional de Engenharia e Tecnologia Industrial (Lisboa, Portugal); CBS, Centraalbureau voor Schimmelcultures (Baarn, the Netherlands).

The cell suspensions were obtained as described above and mixed cultures were prepared by mixing in equal proportions, *Z. bailii* IGC 4806 and *S. cerevisiae* IGC 4072; *Z. bailii* IGC 4806, *P. membranaefaciens* IGC 2487, and *D. anomala* IGC 5133. Decimal dilutions of these suspensions were prepared in sterile deionized water. Then 0.1 ml of the selected dilutions was filtered, in triplicate, through membrane filters with a 0.45-μm pore size (Millipore) with the aid of partial vacuum. In order to obtain a uniform distribution of cells on the surface of the membrane, 50 ml of sterile, deionized water was added to the inoculum (0.1 ml) in the funnel before filtration. The filters were placed onto the surface of plates containing the mentioned culture media and were incubated at 30°C for 96 h before colonies were counted.

**Utilization of the ZBD medium for analysis of the yeast flora in contaminated wines.** Two samples of contaminated Vinho Verde were collected from two containers of a wine cellar in the north of Portugal. Both samples displayed turbidity, apparently due to a refermentation process. Different volumes (0.1, 1.0, and 5.0 ml) of each sample were filtered through a 0.45-μm pore size membrane filter (Millipore) with the application of partial vacuum. In order to obtain a uniform distribution of cells on the surface of the membrane filters, the samples were diluted into 50 ml of sterile deionized water. Membrane duplicates were placed on the surface of YEPD, WLN, WLD, and ZBD medium containing 0.1% (wt/vol) glucose and 0.4% (vol/vol) formic acid and incubated for 96 h at 30°C. After a 4-month refrigeration period at 4°C the two samples of contaminated Vinho Verde were analyzed again by the same procedure.

## RESULTS

**Design of a culture medium for the detection of *Z. bailii*.** The design of the culture medium was based on previous studies demonstrating the ability of *Z. bailii* to use acetic acid in the presence of glucose *(7)*. Thus, the different ability of the various yeast species to grow in a mineral medium containing a sugar and formic acid (mixed-substrate medium) as the only carbon and energy sources was explored. The utilization of the acid is associated with alkalinization; therefore the incorporation of bromocresol green as an acid-base indicator allowed visualization of the different consumption patterns. A species exhibiting a higher acid tolerance and/or consumption rate will lead to a higher alkalinization of the culture medium and, hence, to a color change from green to blue. In this sense, non-*Z. bailii* strains that changed the color of the medium from green to blue were considered as giving a false-positive result.

In order to define experimental conditions where only *Z. bailii* strains would give a positive response, the acid concentration was adjusted to 0.3, 0.4, or 0.5% (vol/vol), while the glucose concentration was maintained at 0.1% (wt/vol). The assays were performed in liquid or solid medium using microplate wells and agar plates, respectively, as summarized in Table 2. Almost all *Z. bailii* strains displayed alkalinization of the mixed culture media after about

TABLE 2. *Alkaline response of different yeast species in liquid (LM) or solid (SM) mixed-substrate mineral medium containing glucose (G, 0.1% wt/vol) and formic acid (FA, 0.3, 0.4, or 0.5%, vol/vol) after 48 h of incubation at 30°C*

| Species | Strain number[a] | No. of strains | G0.1 FA0.3 | | G0.1 FA0.4 | | G0.1 FA0.5 |
|---|---|---|---|---|---|---|---|
| | | | LM | SM | LM | SM | LM |
| *Z. bailii* | | 12 | +[b] | + | + | + | + |
| | ISA 1025 | 1 | + | + | + | + | −[e] |
| | ISA 1095 | 1 | + | −[c] | −[d] | −[d] | −[e] |
| | IGC T5167 | 1 | + | −[c] | −[d] | −[d] | −[e] |
| *Z. bisporus* | | 5 | + | + | + | + | n.d. |
| | | 3 | + | + | −[e] | −[e] | n.d. |
| *Z. rouxii* | | 6 | n.d. | n.d. | − | | n.d. |
| | IGC 4194 | 1 | + | + | −[e] | −[e] | − |
| | ISA 1220 | 1 | − | − | −[e] | −[e] | − |
| *Z. florentinus* | | 1 | − | − | − | − | − |
| *S. bayanus* | | 2 | − | − | − | − | − |
| *S. cerevisiae* | | 21 | − | − | − | − | − |
| *S. pastorianus* | | 2 | − | − | − | − | − |
| *S. ludwigii* | | 3 | n.d. | n.d. | − | − | n.d. |
| *S. pombe* | | 4 | n.d. | n.d. | − | − | n.d. |
| *P. membranaefaciens* | | 13 | − | − | − | − | − |
| *P. anomala* | | 7 | n.d. | n.d. | − | − | n.d. |
| *D. anomala* | | 3 | n.d. | n.d. | − | − | n.d. |
| *D. bruxellensis* | | 4 | n.d. | n.d. | − | − | n.d. |
| *D. hansenii* | | 2 | − | − | − | − | − |
| *I. orientalis* | | 6 | n.d. | n.d. | − | − | n.d. |
| *K. marxianus* | | 5 | n.d. | n.d. | − | − | n.d. |
| *K. apiculata* | | 1 | n.d. | n.d. | − | − | n.d. |
| *L. elongisporus* | | 2 | n.d. | n.d. | − | − | n.d. |
| *R. mucilaginosa* | | 2 | n.d. | n.d. | − | − | n.d. |
| *T. delbrueckii* | | 6 | n.d. | n.d. | − | − | n.d. |

[a] For each species the number of the strains, other than those specified, are referred to in Table 1.
[b] +, positive response (alkalinization, color change of the culture medium); −, negative response (no color change of the culture medium); n.d., not determined.
[c] Alkalinization after an additional incubation period of 24 h.
[d] Alkalinization after an additional incubation period of 24 to 48 h.
[e] Alkalinization after an additional incubation period of 72 to 96 h.

48 h incubation. However, three strains (ISA 1025, ISA 1095, and IGC T5167) changed the color of the medium only after an additional incubation time, which was related with the increase in formic acid concentration. Therefore, they were considered as displaying a slow response.

No major differences were detected in the results from liquid or solid medium, because similar patterns in color change were obtained. All *Zygosaccharomyces bisporus* strains tested were also able to change the color of the mixed culture medium containing 0.3% (vol/vol) formic acid. An increase in the acid concentration to 0.4% (vol/vol) resulted in a prolonged response time (120 h) for three strains of *Z. bisporus*. Two strains of *Zygosaccharomyces rouxii* (IGC 4194 and ISA 1220) exhibited false-positive results in the mixed-substrate medium containing 0.3% (vol/vol) formic acid. However, the increase in acid concentration to 0.5% (vol/vol) eliminated those responses, even after a prolonged incubation time (120 h). All the other yeast species were unable to increase the pH of the culture media and, therefore, to change the color of the medium.

**Validation of the culture medium—recovery of *Z. bailii* cells.** The applicability of the ZBD medium for the microbiological control of wines was evaluated by estimating the percentage recovery of *Z. bailii* and other typical wine contamination yeasts. In addition, the ZBD medium was compared with ZBA, described by Erickson *(1)*.

As shown in Table 3, the percentage recovery of *Z. bailii* cells in the ZBD medium with 0.2, 0.3, or 0.4% (vol/vol) formic acid and in ZBA did not differ significantly ($P \leq 0.05$) from the value obtained with the reference culture medium (YEPD). The increase in acid concentration up to 0.5% (vol/vol) caused a significant decrease ($P \leq 0.05$) in the number of cells recovered compared with the YEPD medium. Contamination of wine with *Z. bailii* can be associated with other yeast species like *S. cerevisiae, P. membranaefaciens,* or *D. anomala.* Mixed cultures of these species and *Z. bailii* (in equal proportions) were tested in order to simulate a contaminated wine and to determine if the presence of other contaminating yeasts could change the percentage recovery of *Z. bailii.* The results (Table 3) showed that with mixed cultures of *Z. bailii* with *S. cer-*

TABLE 3. *Percent recovery (%) of* Z. bailii *cells in pure or mixed culture with other wine-contaminating yeast species on ZBA and on ZBD medium with different formic acid concentrations, using YEPD as reference medium, after incubation for 96 h at 30°C*

| Strain(s) | | YEPD | ZBA | ZBD culture medium[a] G0.1 FA0.2 | G0.1 FA0.3 | G0.1 FA0.4 | G0.1 FA0.5 |
|---|---|---|---|---|---|---|---|
| *Z. bailii* | IGC 4806 | 100 | 72 | 82 | 78 | 65 | 42[c] |
| *Z. bailii* + | IGC 4806 | 100 | 77 | 82 | 81 | 57 | 35[c] |
| *S. cerevisiae* | IGC 4072 | n.d.[b] | n.d. | n.d. | n.d. | n.d. | n.d. |
| *Z. bailii* + | IGC 4806 | 100 | 82 | 99 | 94 | 67 | 34[c] |
| *P. membranaefaciens* + | IGC 2487 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| *D. anomala* | IGC 5133 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| *S. cerevisiae* | IGC 4072 | 100 | 6[c] | 30[c] | 4[c] | <0.002[c] | <0.002[c] |
| *P. membranaefaciens* | IGC 2487 | 100 | <0.004[c] | 55[c] | 5.9[c] | 0.011[c] | <0.004[c] |
| *D. anomala* | IGC 5133 | 100 | <0.004[c] | <0.004[c] | <0.004[c] | <0.004[c] | <0.004[c] |

[a] G0.1 FA0.2, G0.1 FA0.3, G0.1 FA0.4, and G0.1 FA0.5, mineral medium containing glucose (0.1% wt/vol) and formic acid (0.2, 0.3, 0.4, or 0.5%, vol/vol), respectively.
[b] n.d., not determined.
[c] Significant difference ($P \leq 0.05$) compared to the reference culture medium (YEPD).

*evisiae* or *Z. bailii* with *P. membranaefaciens* and *D. anomala,* the percentage of recovery of *Z. bailii* for the various culture media was very similar to the values obtained for this yeast in pure culture. *S. cerevisiae,* when tested in pure culture, grew to a significantly lesser extent on the mixed-substrate medium when compared to the reference medium YEPD. The percentage recovery of about 30% in the medium containing 0.2% (vol/vol) formic acid dramatically decreased to less than 0.002% when the concentration of formic acid was increased to 0.4% (vol/vol). A similar effect was observed with *P. membranaefaciens.* These results are consistent with the higher selectivity of the medium with increasing concentrations of formic acid. *D. anomala* was unable to grow on any of the tested culture media. The percentage recovery of *S. cerevisiae* obtained on ZBD containing glucose (0.1%, wt/vol) and formic acid (0.2%, vol/vol) was higher than on the ZBA medium. With the increase of acid concentration to 0.4% (vol/vol) in the presence of glucose (0.1%, wt/vol), the recovery rate decreased, being below the value determined for ZBA. The ideal mixed-substrate culture medium composition for the enumeration of *Z. bailii* cells in the presence of other wine-contaminating yeasts appeared to be this latter one.

The color and colony morphology of the four yeasts in mixed-substrate medium containing 0.2% (vol/vol) formic acid is illustrated in Figure 1. The colonies of *Z. bailii* are easily identified by their blue color (Fig. 1A). On the same medium, *S. cerevisiae* formed white, light-green, or green colonies (Fig. 1B), indicating that the acid-base indicator, bromocresol green, might be incorporated into some cells. *P. membranaefaciens* formed dark green colonies (Fig. 1C). Using the mixed cultures, it was possible to distinguish the blue *Z. bailii* colonies from the white *S. cerevisiae* colonies (Fig. 1D). Nevertheless, several colonies were light blue, resulting possibly from *S. cerevisiae* cells that were able to incorporate the acid-base indicator (of blue color due to the alkalinization performed by *Z. bailii*), as was already observed for this yeast when tested in pure culture. *P. membranaefaciens* formed dark blue colonies in the presence of *Z. bailii* (Fig. 1E) that could be distinguished clearly from each other by size and intensity of the blue color.

**Field trial evaluation with the mixed-substrate mineral medium.** In order to validate the differential medium developed, two samples of contaminated Vinho Verde from a wine cellar in the north of Portugal were analyzed by membrane filtration. The number and morphology of the colonies developed on the mixed-substrate medium were compared to other culture media such as WLN and WLD that are commonly used for the microbiological analysis of wines. The results obtained are shown in Table 4. The values refer to a first analysis, performed immediately after sampling, and a second analysis, after refrigeration of the wine for 4 months. Before refrigeration, the number of yeasts able to grow on WLN (the medium normally used for the detection of the total yeast flora in wines) was of the same order of magnitude in the two wines. Major differences were detected, using either WLD (the medium for the enumeration of the nonfermenting flora in wines as well as lactic and acetic acid bacteria) or the ZBD medium containing formic acid (0.4% vol/vol) and glucose (0.1% wt/vol). The number of yeasts able to grow on WLD was 20-fold higher in wine 2 compared with wine 1, suggesting differences in the composition of the yeast population in the two wines. In wine 2, the ZBD medium allowed the detection of 170 blue-colored colonies, indicating the presence of *Z. bailii.* One of those blue colonies was confirmed to be *Z. bailii* by molecular typing. On the same medium, a similar number of slightly yellow–creamy colonies was found in both wines, but they were non-*Z. bailii* species when assayed by molecular typing.

After a 4-month refrigeration, both wines had a similar number of yeasts able to grow on YEPD. The refrigeration period leads to a dramatic decrease in the yeast counts in WLN, resulting in 10% of the initial count for wine 1 and 0.3% for wine 2. No yeasts were able to grow on the mixed-substrate medium (ZBD) after refrigeration.

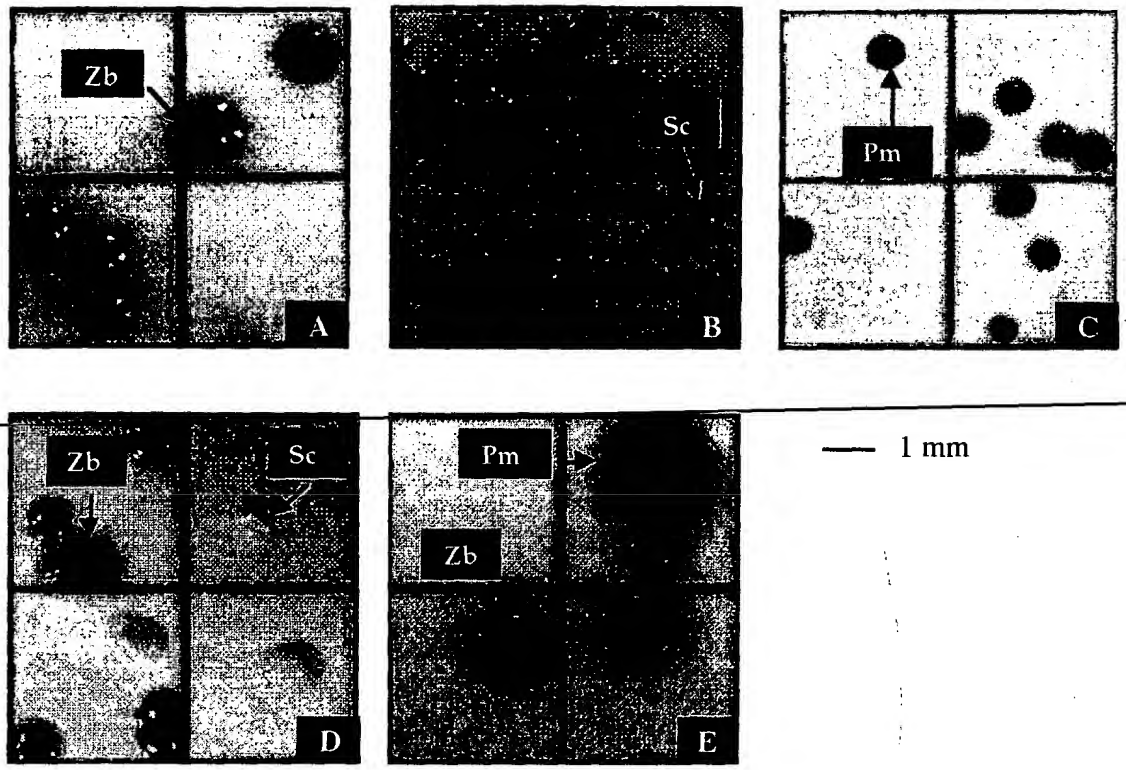


FIGURE 1. *Morphology of the colonies of* Z. bailii *in pure or mixed culture with other wine-contaminating yeasts on membrane filters placed on the surface of the mixed-substrate medium containing formic acid (0.2%, vol/vol) and glucose (0.1%, wt/vol) after incubation for 96 h at 30°C. (A)* Z. bailii *IGC 4806* (Zb)*; (B)* S. cerevisiae *IGC 4072* (Sc)*; (C)* P. membranaefaciens *IGC 2487* (Pm)*; (D)* Z. bailii *IGC 4806* (Zb) + S. cerevisiae *IGC 4072* (Sc)*; (E)* Z. bailii *IGC 4806* (Zb) + P. membranaefaciens *IGC 2487* (Pm).

## DISCUSSION

All 15 strains of *Z. bailii* tested were able to increase the pH of the ZBD medium containing formic acid (0.3%, vol/vol) and glucose (0.1% wt/vol) as carbon and energy sources. Similar results were obtained when the acid concentration was increased up to 0.5% (vol/vol). Although some strains, referred to as slow strains, displayed a prolonged response time, this could be because those strains were more sensitive to the toxic effects of formic acid, yielding a lower growth rate in the mixed-substrate medium and/or a reduced capability to transport and metabolize the acid. However, those strains displaying a faster alkalinization must be considered the most troublesome in a beverage/food production plant. In the assays carried out using ZBD medium, several strains of *Z. bisporus* were also included. Some of these strains exhibited a positive response, indicating that the medium developed is appropriate for the detection of both species. *Z. bisporus* has a similar spoilage potential to *Z. bailii* but can be considered of rather less importance as a wine spoilage yeast. Therefore, these results do not invalidate the application of the ZBD medium for the microbiological control of wines. With formic acid concentrations above 0.3% (vol/vol), none of the other species showed an increase in the pH of the mixed-substrate culture media after 48 h of incubation, indicating lower tolerance of non-*Z. bailii* species to formic acid.

TABLE 4. *Analysis of two samples of contaminated Vinho Verde using the mixed-substrate culture medium ZBD (FA0.4 G0.1) containing formic acid (0.4%, vol/vol) and glucose (0.1%, wt/vol) and YEPD, WLN, and WLD media; the incubation was carried out at 30°C for 96 h and the results are expressed in CFU per ml of wine*

| Culture medium | Wine 1 | | Wine 2 | |
|---|---|---|---|---|
| | Before refrigeration | After refrigeration | Before refrigeration | After refrigeration |
| YEPD | n.d.[a] | 107 | n.d. | 85 |
| WLN | 685 | 73 | 620 | 2 |
| WLD | 10 | 3 | 200 | 2 |
| ZBD: FA0.4 G0.1 | 75[b] | 0 | 90[b] + 170[c] | 0 |

[a] n.d., not determined.
[b] Slightly yellow, creamy colonies.
[c] Blue colonies, typical of *Z. bailii*.

All the assays were carried out in liquid or solid medium. In industrial practice, the media most commonly used are solidified. As the results obtained with the liquid or solid mixed-substrate media were analogous, the validation of the medium was performed with the latter. The percentage recovery of *Z. bailii* obtained with the ZBD medium was similar to that formerly described *(1)*. ZBD was a better differential medium as it allowed a clear distinction between blue colonies formed by *Z. bailii* and colonies of other wine-contaminating species like *S. cerevisiae* and *P. membranaefaciens,* facilitating the interpretation of results. The final composition of the ZBD medium (formic acid, 0.4% vol/vol and glucose, 0.1%, wt/vol) was adjusted as a compromise between recovery and selectivity for *Z. bailii.*

ZBD medium (formic acid, 0.4% vol/vol and glucose, 0.1%, wt/vol) was appropriate for the differential enumeration of *Z. bailii* in two samples of the same wine, originating from different containers (1 and 2). This species was only detected in wine 2, suggesting the contamination occurred in the wine production plant. This is probably due to inadequate sanitation. The yeasts isolated from wine 2 and grown on WLD medium are most probably *Z. bailii* because the number of colonies is of the same order of magnitude as the blue colonies determined in the mixed-substrate medium. Nevertheless, in both wines analyzed, the ZBD medium recovered a higher number of yeast cells than WLD medium before refrigeration.

Refrigeration for 4 months caused a dramatic reduction of the total yeast count in the two samples. Under those conditions, *Z. bailii* colonies were not detected on ZBD. This may be attributed to the loss of cell viability or to an increased sensitivity to formic acid after long-term storage under low temperature stress conditions. After the refrigeration period, the general medium for the isolation of yeasts (YEPD) recovered a higher number of cells than WLN. Therefore, the former medium appears to be a more appropriate culture medium for the detection of yeast cells that have suffered stress during production and/or storage of wines.

The use of the newly described ZBD medium applying the membrane filtration method seems to be the most appropriate for two reasons. First, the white color of the membrane allows good contrast to the blue colonies and therefore a better distinction of white or light-blue colonies formed by *S. cerevisiae* and blue colonies of *Z. bailii,* as illustrated in Figure 1. Second, the low pH of the medium causes a reduced gel strength, so that inoculation by streaking or by spread-plating can be difficult. Nevertheless, the use of a sterile cotton swab instead of an inoculation loop for inoculation by streaking seems to be an acceptable solution.

Interestingly, this medium, with a percentage recovery around 60% for *Z. bailii,* allowed the detection of other wine-contaminating yeast species. A differential medium able to recover most of the wine-contaminating yeast might be achieved by the appropriate manipulation of the formic acid concentration.

In summary, the nature of the acid substrate and manipulation of the pH and of the proportion of the two carbon and energy sources in the mixed medium allowed distinction of different behavior patterns among the various species tested. Based on such differences, and taking advantage of the simultaneous or sequential metabolism of the sugar and the acid, a rapid method for the differentiation of *Z. bailii* was developed. The mixed-substrate medium ZBD allowed differential and simple detection of *Z. bailii* in contaminated wine samples. The inclusion of this medium in the group of media used for the microbiological monitoring of wine may allow the detection of *Z. bailii* and, consequently, adequate implementation of corrective measures when such a contamination is detected.

## ACKNOWLEDGMENTS

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| 2333 | *Zygosaccharomyces bailii* (Lindner) Guilliermond, teleomorph deposited as *Zygosaccharomyces manchuricus* Saito, teleomorph | [NRRL Y-125] | ☐ |
| 2602 | *Zygosaccharomyces bailii* (Lindner) Guilliermond, teleomorph deposited as *Zygosaccharomyces manchuricus* Saito, teleomorph | [NRRL Y-54] | ☐ |
| 28166 | *Zygosaccharomyces rouxii* (Boutroux) Yarrow, teleomorph deposited as *Saccharomyces bailii* var. *osmophilus* van der Walt, teleomorph | A.W.R.I.742 | ☐ |
| 36946 | *Zygosaccharomyces bailii* (Lindner) Guilliermond, teleomorph deposited as *Saccharomyces acidifaciens* (Nickerson) Lodder et Kreger-van Rij, teleomorph | 1 | ☐ |
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| 56535 | *Zygosaccharomyces bailii* (Lindner) Guilliermond, teleomorph deposited as *Saccharomyces bailii* Lindner, teleomorph | N 2120 | ☐ |
| 58445 | *Zygosaccharomyces bailii* (Lindner) Guilliermond, teleomorph | CBS 680 [CCRC 21525; DBVPG 6287; IFO 1098; IGC 2470; NCYC 1416; NRRL Y-2227] | ☐ |
| 60483 | *Zygosaccharomyces bailii* (Lindner) Guilliermond, teleomorph deposited as *Saccharomyces bailii* Lindner, teleomorph | SG 242 [MB Y-24] | ☐ |
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| 60485 | *Zygosaccharomyces rouxii* (Boutroux) Yarrow, teleomorph deposited as *Saccharomyces bailii* var. *osmophilus* van der Walt, teleomorph | SG 144 [R 1709] | ☐ |
| 66825 | *Zygosaccharomyces bailii* (Lindner) Guilliermond, teleomorph | A022 [IGC 4267; ISA 1022] | ☐ |
| 66826 | *Zygosaccharomyces bailii* (Lindner) Guilliermond, teleomorph | G899 [IGC 2899; ISA 1100] | ☐ |
| 8099 | *Zygosaccharomyces bailii* (Lindner) Guilliermond, teleomorph deposited as *Saccharomyces bailii* Lindner, teleomorph | 651 [NCYC 580] | ☐ |
| 8766 | *Zygosaccharomyces bailii* (Lindner) Guilliermond, teleomorph deposited as *Zygosaccharomyces acidifaciens* Nickerson, teleomorph | [BI CZAS 327; CBS 749; CCY 21-27-1; DBVPG 6380; IFO 0722; IFO 1126; IFO 1137; NCYC 573; NRRL Y-1011] | ☐ |

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| 22694 | *Zygosaccharomyces bisporus* Naganishi, teleomorph deposited as *Saccharomyces bisporus* (Naganishi) Lodder et Kreger-van Rij, teleomorph | M 189 [IFO 1736; KCM 0233; NRRL Y-408] | ☐ |
| 2607 | *Zygosaccharomyces bisporus* Naganishi, teleomorph | [IFO 1737; KCM 0235; NRRL Y-223] | ☐ |
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| 34890 | *Zygosaccharomyces rouxii* (Boutroux) Yarrow, teleomorph deposited as *Saccharomyces bisporus* var. *mellis* (Fabian et Quinet) van der Walt, teleomorph | NARD 3344 [CBS 6683] | ☐ |
| 38993 | *Zygosaccharomyces bisporus* Naganishi, teleomorph deposited as *Saccharomyces bisporus* (Naganishi) Lodder et Kreger-van Rij var. *bisporus*, teleomorph | Y-2 | ☐ |
| 46246 | *Zygosaccharomyces rouxii* (Boutroux) Yarrow, teleomorph deposited as *Saccharomyces bisporus* (Naganishi) Lodder et Kreger-van Rij, teleomorph | NRRL YB-4810 | ☐ |
| 52405 | *Zygosaccharomyces bisporus* Naganishi, teleomorph | CBS 702 [CCRC 21505; DBVPG 6382; IFO 1131; NCYC 1495; NRRL Y-12626; NRRL Y-7558; NRRL Y-7683; UCD 66-24] | ☐ |
| 52406 | *Zygosaccharomyces bisporus* Naganishi, teleomorph | CBS 1082 [CCRC 21726; DBVPG 6383; IFO 1250; NRRL Y-12627; VKM Y-352] | ☐ |
| 52407 | *Zygosaccharomyces bisporus* Naganishi, teleomorph | CBS 1083 [CCRC 21725; DBVPG 6384; IFO 1249; NRRL Y-7684] | ☐ |
| 52428 | *Zygosaccharomyces bisporus* Naganishi, teleomorph deposited as *Saccharomyces bisporus* (Naganishi) Lodder et Kreger-van Rij, | TFY 1 | ☐ |

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|---|---|---|---|
| | teleomorph | | |
| 60348 | *Zygosaccharomyces bisporus* Naganishi, teleomorph deposited as *Saccharomyces bisporus* (Naganishi) Lodder et Kreger-van Rij, teleomorph | AWRI 114 (366) | ☐ |
| 64332 | *Zygosaccharomyces bisporus* Naganishi, teleomorph deposited as *Saccharomyces bisporus* (Naganishi) Lodder et Kreger-van Rij, teleomorph | H53 [CCY 21-23-2] | ☐ |
| 8381 | *Zygosaccharomyces rouxii* (Boutroux) Yarrow, teleomorph deposited as *Saccharomyces bisporus* (Naganishi) Lodder et Kreger-van Rij var. *bisporus*, teleomorph | NRRL 1219 [CBS 742; IFO 1733; KCM 0236; M1] | ☐ |

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